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FOREWORD

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_S. Thakur_____

PI - Signature   **Date**

Table of Contents

Annual Summary Report

The principal aim of my project was to characterize the transcriptional regulation of the breast cancer susceptibility gene, BRCA1. This purpose of the project has gained greater importance in light the following observations by several laboratories: (1) A significant number of higher grade breast cancers have decreased expression of BRCA1 mRNA and proteins and (2) BRCA1 transcriptional promoters have been reported to be methylated in some sporadic breast cancers as opposed to lack of metylation in normal breast tissues. These studies suggested that suppression of BRCA1 transcription may contribute to the development of a subset of breast cancer. Therefore, the present studies may not only give greater insight into the functional pathways in which BRCA1 participates in, but also provide clues regarding the pathogenesis of breast neoplasms.

In our studies, we identified a positive regulatory region (PRR) in the BRCA1 promoter. Deletion of the PRR resulted in a significant loss of BRCA1 promoter activity. In addition, proteins with specific binding affinities were observed to bind the PRR. These studies were published in J. Biol. Chem and a copy of the publication is enclosed herewith. Further studies indicated that the PRR consists of two discrete functional domains- (i) A homopolypyrimidine/polypurine (Py/Pu) tract and (ii) A CREB- (cAMP response element binding) like binding site. Each domain possesses specific affinity for distinct factors. Subtle mutations generated in the PRR diminishes the protein binding affinity and is accompanied by a correlative loss in transcriptional activity of the BRCA1 promoter. Studies also suggest that the binding of transcription factors to the PRR is predominant in cells arrested in late G1 phase. The DNA binding is decreased in cells arrested in the S phase and is significantly decreased in cells inhibited in the M phase. The data hints that the cell-cycle specific regulation of BRCA1 transcription is mediated by the PRR. Overall, the results reinforce the important role of the PRR in BRCA1 transcription, and suggest that the inhibition of PRR function may contribute to decreased BRCA1 expression, perhaps contributing to increased risk of breast cancer susceptibility. We are in the process of submitting a manuscript based on these results. In addition, we have initiated projects to identify the transcription factors which bind the PRR.

Principal Investigator: Sanjay Thakur Ph.D.

Positive Regulation of the BRCA1 Promoter*

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Inherited mutations in the *BRCA1* gene, presumably leading to loss of function, confer susceptibility to breast and ovarian neoplasms and are thought to be responsible for approximately 2.5–5% of all breast cancers. It has been suggested that alternative mechanisms, such as disruption of transcription, may also be involved in the suppression of *BRCA1* gene expression/function in breast cancers. Therefore, we initiated studies on the *BRCA1* transcriptional promoter. Utilizing systematic promoter deletions and transient transfection assays, a 36-base pair region was determined to be important for the positive regulation of *BRCA1* transcription. Deletion of this positive regulatory region resulted in a significant loss of promoter activity. Utilizing DNA binding assays, proteins with specific affinities for the positive regulatory region were detected. Disruption of the DNA-protein complexes could affect normal *BRCA1* transcription and may contribute to breast cancer susceptibility.

Breast cancer is the second leading cause of death in American women, accounting for more than 50,000 deaths each year. Current estimates place the average American woman's lifetime risk of developing breast cancer at approximately 11%. However, women with two or more first degree relatives with breast cancer have an estimated 13-fold increased risk over the general population (1). Breast cancer in such families has an inheritance pattern consistent with a highly penetrant autosomal dominant allele (2, 3). *BRCA1*, the first breast cancer susceptibility gene to be identified (4), was isolated in 1994 (5).

An interesting fact regarding *BRCA1* is that although the mutations in the gene in familial breast cancers are of high penetrance, very few mutations in the *BRCA1* gene have been found in sporadic forms of the cancer. These findings are prompting researchers to study the possibility of disruption of *BRCA1* function through epigenetic mechanisms. Consistent with this notion, it has been suggested that transcriptional dysregulation of *BRCA1* may play a role in suppressing *BRCA1* expression in breast cells, perhaps contributing to the development of a neoplastic phenotype. Two studies have demonstrated a decrease of *BRCA1* expression in sporadic breast cancer (6, 7). Another set of studies describe CpG methylation of *BRCA1* transcriptional promoter in a number of sporadic

breast cancers, in contrast to the lack of methylation in normal breast tissues samples (8, 9).

In light of these observations, we initiated a study to characterize the *BRCA1* transcriptional promoter. Previously, the structural features of the *BRCA1* promoter were described (10, 11), and preliminary descriptions of modest and indirect effects of estrogen on the *BRCA1* promoter activity were reported (12, 13). However, no information was available regarding regulatory sites and specific regulatory factors. In this report we provide evidence for a positive regulatory region (PRR)[1] in the *BRCA1* promoter and show data that suggest that multiple proteins bind specifically to the site.

EXPERIMENTAL PROCEDURES

Isolation of the BRCA1 Promoter—The *BRCA1* promoter was subcloned from a bacterial artificial chromosome clone, BAC 694 (kindly provided by Dr. Sean Tavtigian, Myriad Genetics) (14). Briefly, *Pst*I linker (5′-GCTGCAGC-3′) was ligated into the blunted *Hin*dIII site in the pGL2 vector (Promega vector with the firefly luciferase reporter gene). BAC 694 was digested with *Pst*I, and the resulting fragments were shotgun cloned into the pGL2 vector and transformed into competent DH5-α *Escherichia coli* cells. The transformed bacterial colonies were screened by colony hybridization with a radiolabeled *BRCA1* cDNA probe (*BRCA1* cDNA was kindly provided by Frank Calzone) (15) labeled by the random hexamer method (16), and clones with a 3.8-kilobase insert containing the *BRCA1* 5′ genomic fragment were selected. The cloned fragment was sequenced completely and is identical to the previously described genomic fragment encompassing the *BRCA1* promoter (GenBank™ accession number U37574) (11). The nucleotide position of mutants are numbers from the P1 promoter initiation site at nucleotide 1582.

Mutants of BRCA1 Promoter—Systematic promoter deletions were constructed by unidirectional exonuclease III digestion. 10 µg of *BRCA1* promoter-luciferase construct was digested with *Mlu*I restriction enzyme and blunted with α-phosphorothioate nucleotides using Klenow enzyme. This treatment rendered the ends of the linearized plasmid resistant to exonuclease III digestion. The linearized DNA was purified by phenol/chloroform/isoamyl alcohol extraction and digested with *Xho*I restriction enzyme, generating a 5′ end susceptible to the exonuclease digestion. The fragment was purified and then subjected to exonuclease III digestion. Aliquots of the reaction were removed at regular intervals, and the reactions were terminated. Finally, the fragments were blunted using S1 nuclease, religated, and transformed into DH5-α competent cells. DNA preparations (using Qiagen columns) made from selected colonies were screened by analytical restriction enzyme digestions. Mutants −202 and +20 were constructed by exploiting the restriction enzyme sites present on the *BRCA1* promoter (*Eco*RI, −202; *Sac*I, +20) and also the sites present in the polylinker of the luciferase vector. The *BRCA1*-luciferase construct was digested with *Eco*RI/*Xho*I (*Xho*I site is present in the polylinker) and *Sac*I (*Sac*I site is also present in the polylinker), respectively, and blunted with Klenow enzyme, and the larger fragment gel was purified and religated.

Additional mutants to generate progressive deletions were constructed by a polymerase chain reaction based strategy. 5′ primers were designed at regular intervals along the sequence of the *BRCA1* promoter: −245, 5′-CTC ACG CGT TAG AGG CTA GAG GGC AGG-3′; −198, 5′-CTC ACG CGT TCC TCT TCC GTC TCT TTC-3′; −177,

[1] The abbreviations used are: PRR, positive regulatory region; BrdUrd, 5-bromo-2′-deoxyuridine; Py-Pu, polypyrimidine-polypurine; CREB, cAMP response element-binding protein.

5'-CTC ACG CGT TTA CGT CAT CCG GGG GCA-3'; −162, 5'-CTC ACG CGT GCA GAC TGG GTG GCC AAT-3'; −152, 5'-CTC ACG CGT TGG CCA ATC CAG AGC CCC-3'; −118, 5'-CTC ACG CGT CTT TCT GTC CCT CCC ATC-3'; −86, 5'-CTC ACG CGT GAT TTC GTA TTC TGA GAG-3'; −43, 5'-CTC ACG CGT GGT TTC CGT GGC AAC GGA-3'; −34, 5'-CTC ACG CGT GGC AAC GGA AAA GCG CGG-3'; −26, 5'-CTC ACG CGT AAA GCG CGG GAA TTA CAG-3'; −17, 5'-CTC ACG CGT GAA TTA CAG ATA AAT TAA-3'; −7, 5'-CTC ACG CGT TAA ATT AAA ACT GCG ACT-3'. The 5' primers included three bases, CTC, followed by the *Mlu*I enzyme site, which is underlined. The single 3' primer used was: +36, 5'-TAG CTC GAG GGA AGT CTC AGC GAG CTC-3'. This primer included TAG followed by a *Xho*I site, which is underlined, at the 5' end. The amplification conditions used were as follows (1 cycle for 2 min at 94 °C and 35 cycles at 94 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s), and 1 ng of the cloned *BRCA1* promoter plasmid was used as template DNA. The amplified products were digested with *Mlu*I and *Xho*I restriction enzymes and ligated into the restricted *Mlu*I-*Xho*I site of the pGL3 basic vectors (Promega). In addition, two synthetic primers (+5, 5'-CGC GTT GCG ACT GCG CGG CGT GAG CTC GCT GAG ACT TCC TC-3' and +36, 5'-TCG AGA GGA AGT CTC AGC GAG CTC ACG CCG CGC AGT CGC AA-3') designed to include the *BRCA1* promoter region from +5 to +36 were annealed. The annealed primers generated a *Mlu*I compatible site at the 5' end and a *Xho*I compatible site at the 3' end, which were utilized in ligation of the annealed primers directly into the *Mlu*I and *Xho*I site of the pGL3 vector. All the constructs were verified by sequencing.

Cell Culture, Transfections, and Reporter Gene Assays—TK-TS13 cells (hamster kidney cells kindly provided by Dr. Bruno Calabretta) were transfected by calcium phosphate precipitation. A total of 3 μg of DNA was used in each transfection (1 μg of BRCA promoter, 1 μg of pUC19 plasmid, and 1 μg of pRL-CMV Renilla luciferase vector purchased from Promega). The cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and 100 μg/liter gentamycin sulfate. MCF-7 cells (maintained in minimum essential medium, 10% fetal bovine serum, 100 μg/ml gentamycin sulfate, 2 mM sodium pyruvate and glutamine) were transfected using Fugene-6 reagent manufactured by Boehringer Mannheim. A total of 2 μg of DNA (0.5 μg of promoter construct and 1.5 μg of pRL-CMV) with 10 μl of Fugene reagent was used in each transfection. All the transfections were optimized for six-well plates. The transfected cells were lysed after 48 h by addition of 250 μl of passive lysis buffer (Promega). 20 μl of the lysed cell extract was added to 100 μl of luciferase substrate, and the light emissions were measured in a scintillation counter. Renilla luciferase readings (which were utilized to normalize the transfection efficiencies) were measured in the same tube using conditions recommended by the manufacturer.

DNA Binding Assays—The experiments were performed essentially as described previously (17). Electrophoretic mobility shift assays were performed with a double-stranded probe encompassing the PRR (see Fig. 3*B*). Two single-stranded oligonucleotides (−197 to −161, 5'-CCT CTT CCG TCT CTT TCC TTT TAC GTC ATC CGG GGG CAG ACT-3', and −161 to −171, 5'-AGT CTG CCC CC-3') were annealed, and the gap was filled by Klenow polymerase in the presence of deoxyribonucleotides dATP, dGTP, dTTP, α-^{32}P-radiolabeled dCTP and 5-bromo-2'-deoxyuridine (BrdUrd) as described (17). Nuclear extract (5 μg), made from MCF-7 breast cancer line as described previously, was mixed with 100,000 cpm of probe in Yanos buffer (20 mM Tris, pH 7.8, 50 mM NaCl, 10 mM MgCl$_2$, 15% glycerol, 0.1 mM EDTA, 0.01% Nonidet P-40, 100 μg/ml bovine serum albumin, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0 or 1.5 or 3 μg of poly(dI·dC)) and incubated for 20 min. Competitions were performed with 50-fold excess of nonradiolabeled annealed double-stranded oligonucleotides. These oligonucleotides contained palindromic C/EBP mutant (5'-TGC AGA GAC TAG TCT CTG CA-3'), p53 (5'-CCC AAA CAA GCT CCC CTG AAA CAA GCC CGT T-3' and 5'-AAC GGG CTT GTT TCA GGG GAG CTT GTT TGG G-3'), and palindromic C/EBP canonical sites (5'-TGC AGA TTG CGC AAT CTG CA-3'). In addition, sheared salmon sperm DNA (Sigma) and PRR cold probes were also used in competitions. After the incubations, loading dye was added (final concentrations: 0.06% bromphenol blue, 0.06% Xylene cyanole, and 7.2% glycerol), and the reactions were electrophoresed in a 5% nondenaturing gel, resolved for 3 h at 200 V in 0.25× TBE buffer, and after drying the gel exposed for 5 h to a film.

In experiments involving UV radiation-induced cross-linking of DNA and protein, each electrophoretic mobility shift assay reaction was transferred into a 96-well plate at 4 °C and exposed to UV radiation in a UV-Stratalinker 1800 (Stratagene) for 20 min. After the addition of sample buffer (1 M Tris, pH 6.8, 10% SDS, 50% glycerol, 10% β-mer-

captoethanol, and 0.2% bromphenol blue) and boiling for 5 min, the reaction was resolved in a 9% SDS-polyacrylamide gel. The dried gel was exposed to film. Negative control experiments with a random probe were also performed. The random probe was synthesized (in a manner similar to the PRR probe) by the extension of a DNA oligonucleotide (5'-CCC GGG AGT AGA-3') annealed to a longer complementary oligonucleotide (5'-CTA GTC AGA CAC GTA GAC TCT ACT CCC GGG-3') in the presence of BrdUrd and dCTP containing α-^{32}P label.

RESULTS

BRCA1 Minimal Promoter—In order to identify the minimal promoter containing the essential regulatory regions, systematically deleted mutants of the *BRCA1* promoter region were constructed (Fig. 1). The transcriptional activities of these mutants were tested in TK-TS13 and MCF-7 cells by luciferase reporter gene assays (Fig. 2). The TK-TS13 cells were used in initial studies (Fig. 2*A*), due to the ease with which they are transfected and because of their ability to support high levels of *BRCA1* promoter activities. Subsequent detailed studies were performed in MCF-7 breast epithelial cell line (Figs. 2*B* and 3*A*).

Results of transfections of the mutants in TK-TS13 cell indicated that on the deletion of 1380 bases from mutant −1582 (and generating −202), there is a 2.5-fold drop of luciferase activity, which is not significant considering the high sensitivity of the luciferase assays and the number of bases deleted (Fig. 2*A*). Within the same tract of promoter DNA (−1582 to −202), the two most wide ranging luciferase values were 6127 (for −1244) and 308 (for −329) normalized light units, a difference of 20-fold. However, this difference was accompanied by a loss of 915 bases from −1244.

Overall, the data suggest that short deletions within the segment −1582 to −202 do not cause a significant change in promoter activity. However, gross deletions (−1582 to −202) do alter the configuration of the promoter sufficiently to affect the promoter activities significantly, because several weak regulatory sites (both enhancers and repressors) with additive effects may be deleted.

Transfections of selected *BRCA1* promoter mutants in MCF-7 cells also indicated that essential transcriptional regulatory sites (which could have a strong effect on the transcription of *BRCA1*) were not present in the tract from −1582 to −202 (Fig. 2*B*). Interestingly, in MCF-7 cells mutant −202 was observed to possess a transcriptional activity that was comparable (and slightly higher) to that of construct −1582.

Finally, results from both cell lines indicated the presence of a sensitive region of 222 bases (−202 to +20), which on deletion resulted in a 100% loss of *BRCA1* promoter activities (Fig. 2). These experiments strongly suggested that the essential regulatory elements of the *BRCA1* promoter reside within the deleted segment. Furthermore, the mapped segment encompassed the P1 promoter region, suggesting that the activity of the P1 promoter was predominant in both the transfected cell lines. Curiously, P2 did not demonstrate any functional activity in either of the cell lines tested (Fig. 2). It is possible that sequences within the P1 promoter region may regulate the transcriptional initiation from P2.

Identification of the BRCA1 PRR—Following the identification of the −202 to +20 segment as essential for *BRCA1* transcription, detailed characterization of the segment was undertaken. Additional unidirectional, polymerase chain reaction-based deletion mutants were constructed, and their activities were tested (Fig. 3*A*). With the aid of these promoter mutants, it was determined that deletion of a short 22-base pair region between −198 and −177 (Fig. 3*B*) resulted in a significant loss (14.5-fold) of luciferase activity. Further removal of 15 more nucleotides (−162) led to an additional 4-fold loss in activity.
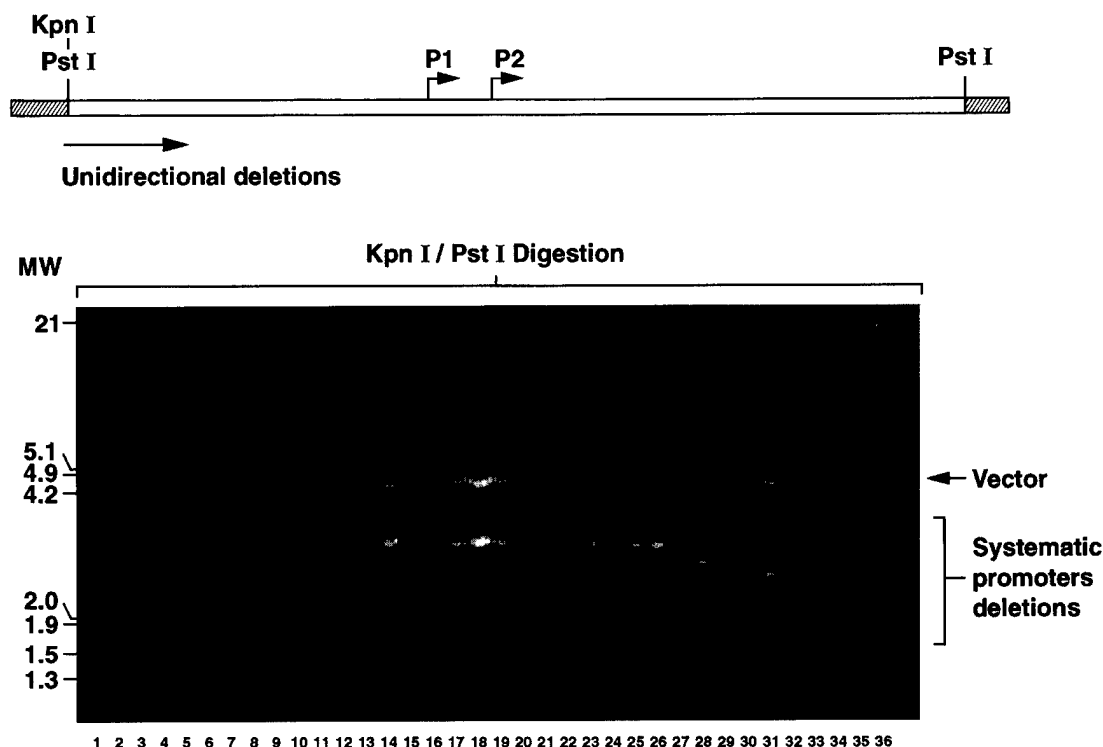
FIG. 1. **BRCA1 promoter region and mutants.** The *upper panel* depicts the *BRCA1* promoter region, with two previously described initiation sites P1 (nucleotide 1582) and P2 (nucleotide 1858) (11). The *lower panel* depicts the generation of unidirectional deletions within the promoter region, which leave the upstream *Kpn*I site in the polylinker intact (the adjacent *Pst*I is within the *BRCA1* promoter). Cleavage of the digested fragment with *Kpn*I and *Pst*I releases the promoter fragment. The numbers of the mutants correspond to the 5' deletions catalogued and characterized in Fig. 2A.

Overall, the removal of 36 bases (from −198 to −162) results in a 56-fold loss in luciferase activity, indicating a PRR.

Interestingly, the PRR contains a short polypyrimidine-polypurine (Py-Pu) tract (the majority of nucleotides on the sense strand are pyrimidines and by extension the complementary strand is mostly purine) (Fig. 3B). The first 22 bases in the site are almost exclusively Py-Pu (21 of 22 or 95%). This Py-Pu-rich tract is followed by a putative CREB site. Overall the pyrimidines in the sense strand make up 75% (27 of 36) of PRR.

DNA Binding Assays—In order to characterize the proteins binding to the PRR site, electrophoretic mobility shift assays were performed using MCF-7 nuclear extracts. Retarded protein-DNA complexes were detected (Fig. 4A, lanes 2–8). Lane 2 lacked poly(dI·dC), and therefore the factors binding the probe largely represented nonspecific proteins. Addition of poly(dI·dC) (*lanes 3–9*), cleared nonspecific bands, and three protein-DNA complexes were detected (except in *lane 9*); a single intense, higher mobility band and two weaker bands with lower mobility, were observed. Addition of 50-fold excess of double-stranded nonlabeled oligonucleotides or sheared salmon sperm DNA (*lanes 5–8*) did not compete away the protein-DNA complexes. In sharp contrast, the proteins binding the labeled probe were efficiently competed out by a 50-fold excess of nonlabeled PRR probe (*lane 9*). This experiment indicates that the PRR-binding proteins bind in a sequence-specific manner.

In order to further characterize the components of the protein complex that assembled on the PRR, UV cross-linking experiments (involving DNA-protein linkage) were performed (Fig. 4B). This involved incubation of radiolabeled, BrdUrd-containing PRR probe, with MCF-7 nuclear extracts in the presence of UV radiation. The radiation induced covalent linking of proteins to the BrdUrd residues present in the PRR probe. All the reactions contained 3 µg of poly(dI·dC), and one

of the reactions was supplemented with 0.5 µg of double-stranded oligonucleotides to increase the stringency of binding to the probe (*lane 3*). Lane 2 exhibits three diffuse and indistinct bands. The intensity of these bands increased on the addition of 0.5 µg of double-stranded oligonucleotides (*lane 3*), reinforcing the possibility that they represent specific protein-DNA interactions. The approximate molecular masses of the protein-DNA complexes observed were 55, 150, and 230 kDa.

Additional experiments were performed to exclude the possibility that the covalent protein-DNA complexes were formed due to nonspecific interactions. A random oligonucleotide labeled with α-^{32}P and BrdUrd residues was used in UV cross-linking experiments (Fig. 4C). Lane 2 of the figure demonstrates the characteristic three-band pattern representing proteins bound to the PRR. In contrast, no significant protein-DNA complexes were observed when the random probe was incubated with MCF-7 nuclear extract (*lane 4*). This experiment suggests that the proteins detected by cross-linking experiments recognized and bound the PRR specifically.
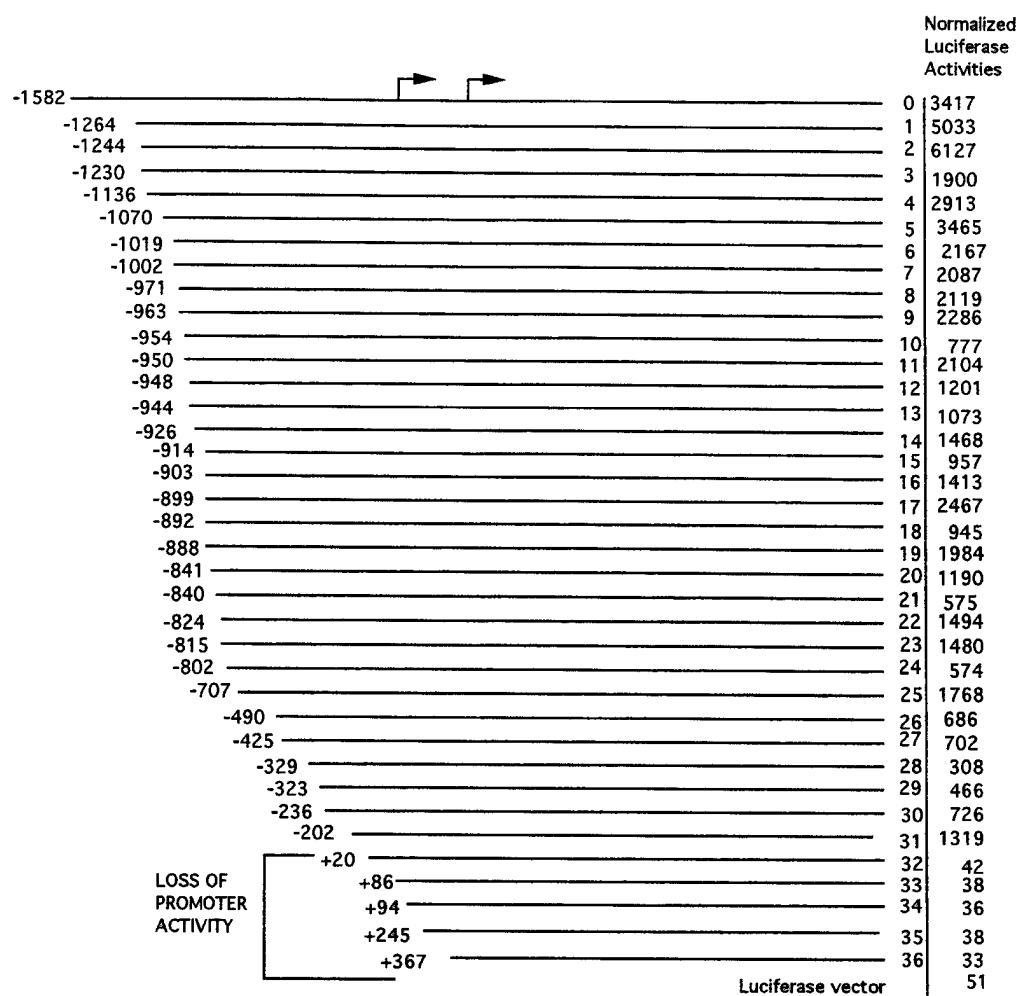
Finally, it is probable that each of the DNA-protein complexes observed do not represent multiple proteins linked to a single DNA probe but a single protein molecule bound to one molecule of the PRR probe. This conclusion is based on the fact that the UV-induced protein-DNA cross-linking is inefficient; therefore it is unlikely that more than one protein molecule will link to a single molecule of DNA probe.
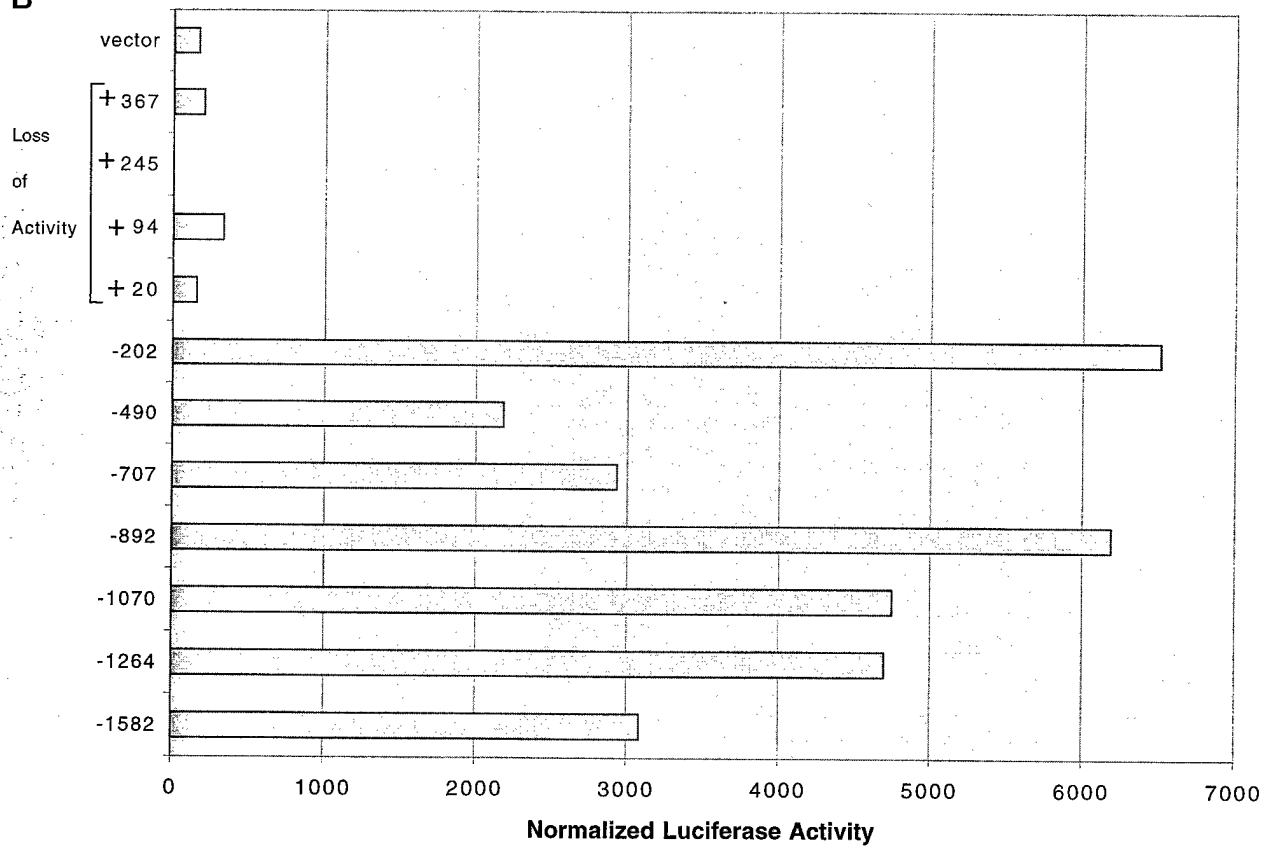
DISCUSSION

Evidence for important roles of *BRCA1* in normal functioning of cells is accumulating. Specifically, a strong role for *BRCA1* in DNA repair mechanisms (18–20) as well as in transcriptional regulation (21–23) has been suggested. This theme is reinforced in a recent report that cites the involvement of *BRCA1* in transcription-coupled repair of DNA (24). Therefore,

Regulation of BRCA1 Transcription

A

Normalized
Luciferase
Activities

-1582	0	3417
-1264	1	5033
-1244	2	6127
-1230	3	1900
-1136	4	2913
-1070	5	3465
-1019	6	2167
-1002	7	2087
-971	8	2119
-963	9	2286
-954	10	777
-950	11	2104
-948	12	1201
-944	13	1073
-926	14	1468
-914	15	957
-903	16	1413
-899	17	2467
-892	18	945
-888	19	1984
-841	20	1190
-840	21	575
-824	22	1494
-815	23	1480
-802	24	574
-707	25	1768
-490	26	686
-425	27	702
-329	28	308
-323	29	466
-236	30	726
-202	31	1319
+20	32	42
+86	33	38
+94	34	36
+245	35	38
+367	36	33

LOSS OF
PROMOTER
ACTIVITY

Luciferase vector | 51

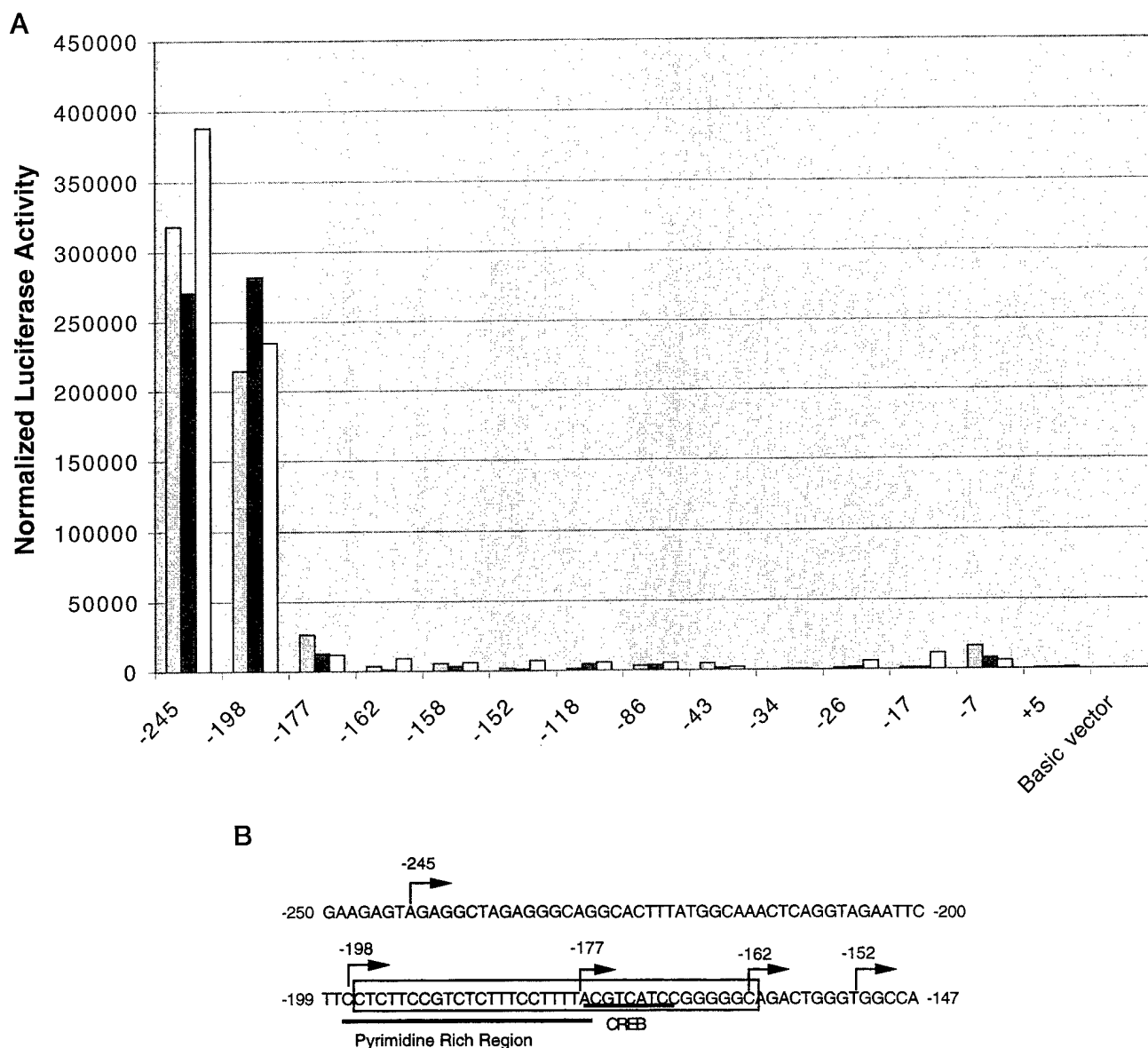**B**

Normalized Luciferase Activity

FIG. 3. **PRR in the *BRCA1* promoter.** *A*, transfections of polymerase chain reaction-generated *BRCA1* promoter mutants with short deletions in the minimal promoter region were performed. All the mutants had the 3' end at nucleotide +36 of the transcription start site. The 5' ends of each mutant are indicated. The data are representative of four independent transfections performed in triplicate for each sample. *B*, the 5' ends of the deletion mutants in close proximity to the PRR (which is *boxed*). The pyrimidine-rich region and the putative CREB site are indicated.

it has been suggested that suppression of *BRCA1* expression may cause defects in the DNA repair machinery, leading to chromosomal defects and tumorigenesis.
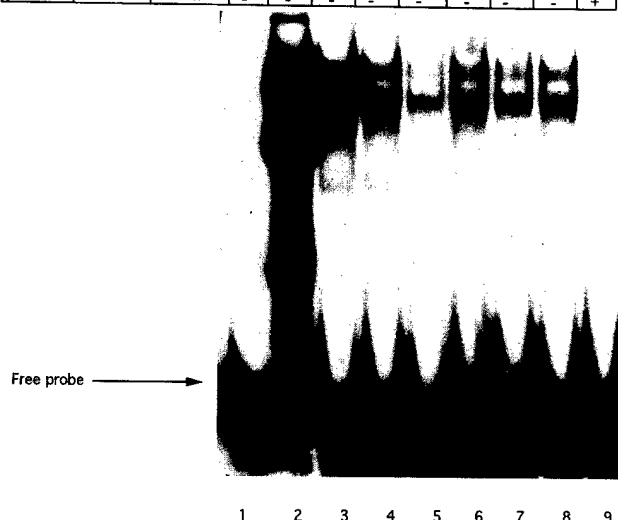
BRCA1 expression may be suppressed through transcriptional silencing in a subset of sporadic breast cancers, underscoring the importance of studies to elucidate the transcriptional mechanisms involved in the regulation of *BRCA1* expression. The present studies strongly suggest that intact and functional PRR may be crucial for normal transcription of *BRCA1*. Hindrance of the PRR function may occur either by methylation of proximate sequences or by alterations in the properties of the regulatory factors, leading to suppression of *BRCA1* expression. Furthermore, elucidation of the factors that regulate *BRCA1* transcription could provide additional clues regarding its function.

It is interesting that the PRR encompasses a CpG dinucleotides reported by Mancini *et al.* (9) to be methylated in one case of sporadic breast cancer. The methylated cytosine was present in the putative CREB site present in the PRR (Fig. 3*B*). CREB proteins are known to mediate hormone stimulation of a variety of genes (25, 26), and *BRCA1* is known to be indirectly responsive to estrogen stimulation (27), prompting speculation of a regulatory role of CREB in *BRCA1* transcription. However, treatment of cells with forskolin (a reagent that stimulates post-translational activation of CREB by phosphorylation; Ref. 28) did not show any effect on the activities of transiently transfected *BRCA1* promoter (data not shown). In addition, forskolin treatment and cotransfection of a CREB-binding protein (a CREB coactivator; Refs. 29 and 30) expression plasmid elicited no response from the *BRCA1* promoter (data not

Fig. 2. **Minimal *BRCA1* promoter.** Luciferase activities of the deletion mutants in TK-TS13 (*A*) and MCF-7 cells (*B*). Deletions that result in loss of promoter activities are indicated and are found to be consistent for the two cell lines. The data are representative of three independent transfections for each line.
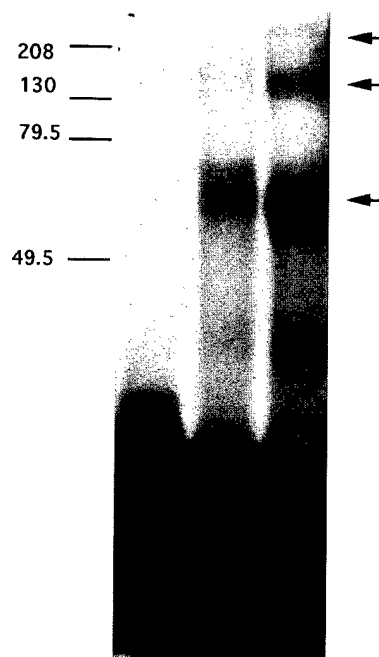
A

Probe	+	+	+	+	+	+	+	+	+
MCF-7 NE	-	+	+	+	+	+	+	+	+
PolydIdC	3 µg	0 µg	1.5µg	3 µg	3 µg	3 µg	3 µg	3 µg	3 µg
CEBP mutant competition	-	-	-	-	+	-	-	-	-
p53 competition	-	-	-	-	-	+	-	-	-
CEBP canonical competition	-	-	-	-	-	-	+	-	-
Sheared SS DNA competition	-	-	-	-	-	-	-	+	-
Specific competition	-	-	-	-	-	-	-	-	+

Free probe →

1   2   3   4   5   6   7   8   9

B

DS Oligo	-	0µg	0.5 µg
PolydIdC	3 µg	3 µg	3 µg
MCF-7 NE	-	+	+
Probe	+	+	+

208 —
130 —
79.5 —
49.5 —

C

Random	-	-	+	+
PRR	+	+	-	-
MCF-7 NE	-	+	-	+

208 —
130 —
79.5 —
49.5 —
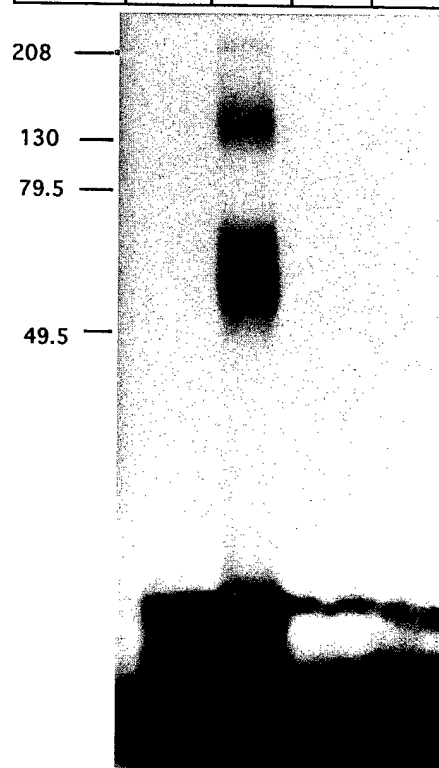
FIG. 4. **Proteins specifically bind the PRR sequence.** *A*, electrophoretic mobility shift assay was performed with a radiolabeled PRR probe. The amounts of poly(dI·dC) added to each reaction are indicated. Nonspecific competitions were performed with 50-fold excess of double-stranded, unlabeled oligonucleotides with indicated binding affinities (*lanes 5–7*). In addition, 50-fold excess of sheared salmon sperm DNA was also used as a nonspecific competitor (*lane 8*). Specific competition was performed by the addition of 50-fold excess of unlabeled PRR-binding oligonucleotide (*lane 9*). *B*, DNA-protein UV cross-linking experiments with PRR probe and MCF-7 nuclear extracts. The protein-DNA complexes are indicated (*arrows*). The free PRR probe is at the *bottom* of the *lanes*. In *lane 3*, 0.5 µg of double-stranded oligonucleotide (*DS Oligo*) containing the binding site for the p53 protein was added. *C*, comparison of protein affinities of PRR and random probes in UV cross-linking assays. All the reactions contain 3 µg of poly(dI·dC) and 0.5 µg of annealed double-stranded oligonucleotide containing the binding site for the C/EBP proteins.

shown). Attempts to identify CREB proteins in the DNA-protein complex, either by supershift assays or immunoprecipitation (of cross-linked DNA-protein complexes) with CREB antibodies, were not successful (data not shown).

The composition of the PRR (21 of the first 22 bases are pyrimidines on the sense strand) provides possible hints regarding mechanisms involved in transcriptional regulation of *BRCA1*. Previous studies have described the tendency for such Py-Pu domains to form triplex DNA, which influence transcription (31, 32). These tracts have been reported to be sensitive to S-1 nuclease digestion and are believed to influence the conformation of the chromatin assembly in the promoter region. In addition, a nuclear factor has been reported to bind a Py-Pu tract in the c-Ki-*ras* promoter (31). Therefore, it is possible that 1) the Py-Pu domain may alter the chromatin structure of the *BRCA1* promoter region and 2) it may also be involved in specific recognition and binding by transcription factors.

There is no additional information available at present regarding the factors binding the PRR. Studies are being initiated to definitively identify and characterize the binding proteins in order to investigate their effects on *BRCA1* transcription and their potential role in breast cancer.

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